CCLXXII. PREPARATION OF SOME PROTEIN SAMPLES FROM THE FRESH LEAVES OF PLANTS AND THE SULPHUR DISTRIBUTIONS OF THE PREPARATIONS

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BOTH from a general standpoint and from the particular one of nutrition, the compositions of the proteins which are present in the leaves of plants are of very considerable interest. In the present article, methods for determining sulphur-distributions, particularly in their bearing upon the estimations of cystine, cysteine and methionine, are examined and applied to several leaf protein preparations.

The leaf protein preparations numbered 1, 2, 3, 5, 6, 7, 8 and 10 in Table I were made during 1935 at Adelaide, South Australia, from fresh leaf material grown on plots which had not been manured for several months. The leaves were macerated directly [cf. Osborne & Wakeman, 1920; Chibnall & Schryver, 1921; Osborne *et al.* 1921; Chibnall, 1922] in a hand-driven corn mill at room temperature (about 20°) with the solvent intended to carry the protein. Ether-water was normally used as solvent [Chibnall & Schryver, 1921] as it was thought that plasmolysis by the ether might assist the dispersion of the protoplasm. The green juice was squeezed through a cloth, filtered through layers of filter paper (which did not retain the chloroplast material very well), and the protein was flocculated with acid at the apparent isoelectric point (about pH 4.5) and coagulated by heating the mixture to about 90° as described by Chibnall *et al.* [1933]. The coagulum was filtered off, washed with 0.0005 *N* HCl and, further to remove impurities, extracted successively with boiling alcohol, boiling 0.005 *M* citric acid (which usually reduces the ultimate ash content but removes only a negligible amount of nitrogenous substance), twice with boiling alcohol (or until extract was almost free from chlorophyll) and finally with warm ether.

In cases 3 and 6, some of the leaf material was ground with 10% aqueous NaCl saturated with ether, instead of with ether-water as were the corresponding preparations, 2 and 5 respectively. Prep. 8 was made by extracting, but not grinding, with this solvent the leaf residues after extraction of 7. The object of using NaCl solution was to provide a satisfactory solvent for globulins in case they happened to be present. Microscopical examination of all the leaf residues revealed that large numbers of the cells had not been ruptured.

Prep. 9 had been made some years ago in this laboratory by the "used ether-water" method of Chibnall *et al.* [1933]. Prep. 4 was made by the same method from leaf material growing on a well-manured plot. The leaf material was first plasmolysed with ether-water which had already been used once for this purpose,

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the readily diffusible constituents were removed by alternate imbibition of water and expression in a Büchner press, and the residue was then macerated with water in a motor-driven mincer. After squeezing the juice through cloth and filtering through a thick pad of paper pulp the protein was flocculated at about pH 4.5 and coagulated by heat. Impurities were removed from the coagulum by extracting with boiling water, alcohol, acetone and ether. The filtered leaf-juice was almost devoid of green colour, suggesting removal of most of the chloroplast material. Many of the leaf cells were not ruptured by the grinding.

The proteins were all denatured in the course of preparation. As regards the proteins properly associated with the plastids and the nuclei, it is improbable that they are adequately represented in preparations obtained by either of these procedures.

In Table I are shown, in the order of the columns: the number distinguishing the preparation: the plant species and date of extraction: the % dry wt. of the leaf material: the % total N (*t*), "albuminoid" N (*a*) and coagulable N (*c*) in the

Table I

No.	Source and date	% dry wt.	% N in dry leaf (<i>t</i> , <i>a</i> , <i>c</i>)	Extraction: Protein-N as % of leaf-N (<i>t</i> , <i>a</i> , <i>c</i>)	% N protein	% ash protein
1	<i>Dactylis glomerata</i> (cocksfoot grass) 3. vi. 35	17.9	2.98 (<i>t</i>), 2.80 (<i>a</i>)	24.0 (<i>t</i>), 25.5 (<i>a</i>)	13.1	3.4
2	" 7. xi. 35	19.0	3.20 (<i>t</i>), 2.82 (<i>a</i>)	24.4 (<i>t</i>), 27.7 (<i>a</i>)	13.0	1.0
3	" 7. xi. 35	19.0	3.20 (<i>t</i>), 2.82 (<i>a</i>)	17.5 (<i>t</i>), 19.9 (<i>a</i>)	13.1	1.0
4	" 29. ix. 36	12.5	6.81 (<i>t</i>), —	26.0 (<i>t</i>), —	14.1	1.1
5	<i>Phalaris tuberosa</i> (Toowoomba canary-grass) 20. viii. 35	20.4	3.08 (<i>t</i>), 2.72 (<i>a</i>)	13.4 (<i>t</i>), 15.2 (<i>a</i>)	15.4	0.7
6	" 20. viii. 35	20.4	3.08 (<i>t</i>), 2.72 (<i>a</i>)	8.1 (<i>t</i>), 9.2 (<i>a</i>)	15.35	0.8
7	<i>Medicago sativa</i> (lucerne) 18. vii. 35	20.1	5.1 (<i>t</i>), 4.4 (<i>a</i>)	27.2 (<i>t</i>), 31.6 (<i>a</i>)	13.85	1.4
8	" 18. vii. 35	20.1	5.1 (<i>t</i>), 4.4 (<i>a</i>)	0.95 (<i>t</i>), 1.1 (<i>a</i>)	13.85	1.7
9	" 29. vi. 33	21.0	3.25 (<i>t</i>), 2.8 (<i>c</i>)	10.9 (<i>t</i>), 12.6 (<i>c</i>)	14.2	1.2
10	<i>Atriplex nummularium</i> (Old-man salt-bush) 6. xii. 35	15.6	3.48 (<i>t</i>), 2.54 (<i>a</i>)	13.1 (<i>t</i>), 17.9 (<i>a</i>)	12.1	4.5

dry-leaf material: the extracted protein-N as a percentage of the leaf-N (*t*, *a*, *c*): the % N in the protein preparation and finally the % of ash in the protein by ignition in air at about 600°. Materials dried in air at 85° were normally used in the analyses. Complete drying (for dry wt.) was done by heating at 105° followed by vacuum desiccation over conc. H₂SO₄. N estimations were made by the Kjeldahl method. The coagulable N was that which was not extracted from the finely-ground dried material at about pH 4.5 by two 10 min. extractions each with 100 times the weight of dilute hydrochloric or acetic acid at 100°, and the albuminoid N was that remaining after extraction under the Stutzer conditions (Cu(OH)₂ reagent).

Methods of determining the sulphur distribution

Assuming provisionally that the only S-containing amino-acids in the protein are cystine (and/or cysteine) and methionine, the distribution may be written: cystine-S + cysteine-S + methionine-S + other S = total S, and if "other S" is present entirely as SO₄ (as inorganic contaminant or attached to —NH₂ groups) and ethereal SO₄ (e.g. of prosthetic groups, if any), it should appear as inorganic SO₄ on prolonged acid hydrolysis at high temperature. In the absence of systematic errors it should thus be possible to check the provisional assumptions to within the limits imposed by the magnitudes of the random errors. The systematic errors, however, may be very large and they are, as yet, so uncertain

in some respects as to make a proper check of the above assumptions impossible. The sources of these errors have been classified roughly elsewhere [Lugg, 1933, 2; 1938] chiefly in relation to specific procedures such as hydrolysis, but it must be realized that sources associated with an assumed extensibility of knowledge of the behaviour of, say, a free amino-acid to the case of the corresponding amino-acid residue in the protein molecule, have an existence independent of procedure. In the following discussions they will be considered in their particular relationships to the estimations concerned.

The most specific methods of estimating cystine and cysteine present in solution appear to be the colorimetric ones. Employed in conjunction with acid (HCl , H_2SO_4) hydrolysis of "pure" proteins they have yielded valuable data, but it is known that errors may be associated with the hydrolysis of impure proteins. Bailey [1937] has shown that the losses resulting from HCl hydrolysis of edestin with arabinose are enormously greater than would be anticipated from hydrolysis tests of free cystine with arabinose and other carbohydrates [Lugg, 1933, 2], but the causes remain obscure. They may be connected with the formation, even temporarily, of sulphhydryl groups by hydrolytic cleavage of disulphide bonds in edestin [Mirsky & Anson, 1934-5; Astbury *et al.* 1935; Mirsky, 1935-6], for in the presence of carbohydrates and their decomposition products the resulting mercaptal and mercaptole formation could effect large losses [Lugg, 1933, 2]. The procedure might be expected to lead to serious underestimation with the impure leaf proteins which normally contain substances of carbohydrate nature. It was found that the losses of cystine could not be averted by hydrolysing with the HCl-H.COOH reagent of Miller & du Vigneaud [1937] in place of HCl . Hydrolysis with alkali-stannite reagents could not be exploited, for whilst free cystine is almost quantitatively reduced to cysteine, edestin yields little more than half the cysteine to correspond with the cystine content of HCl hydrolysates.

Blumenthal & Clarke [1935], in a valuable contribution, estimated the following S-fractions in some protein preparations: sulphate-S after acid hydrolysis, labile S (i.e. appearing as PbS after reduction and digestion with alkali-plumbite), SO_4 -S after oxidation with bromine-water, SO_4 -S after oxidation with HNO_3 and non- SO_4 -S after HNO_3 oxidation (i.e. by difference between total S and the preceding value). They tested the procedures, which do not permit discrimination between cystine and cysteine, with mixtures of cystine and methionine, and adjusted conditions so that the labile S and SO_4 -S due to HNO_3 oxidation would correspond with the cystine present, and the non- SO_4 -S after HNO_3 oxidation with the methionine, for whilst cystine-S is readily oxidized to SO_4 by HNO_3 methionine-S is only slowly oxidized beyond sulfoxide, sulphone or possibly methanesulphonic acid. Neither cystine nor methionine yield much SO_4 when treated with bromine water. As the estimated S-fractions in the proteins were not in complete conformity with the cystine and methionine contents estimated by fairly specific methods and did not show the same interrelationships as mixtures of cystine and methionine would have shown, and were often unaffected by working with HCl hydrolysates of the proteins instead of the proteins themselves, they attributed the discrepancies to the presence of small quantities of other S-containing amino-acids apart from cysteine. Such amino-acids may indeed occur in proteins, but the claim is based upon the assumption that the amino-acid residues behaved in the various procedures precisely as do the free amino-acids, and there is therefore the distinct possibility that unavoidable deficiencies of method suggested the presence of substances that did not exist in the proteins.

$\text{SO}_4\text{-S}$ after HCl hydrolysis, $\text{SO}_4\text{-S}$ after HNO_3 oxidation and total S estimations, which may be regarded as comprising "differential oxidation" data, were all made with the leaf proteins. The other S-fractions studied by Blumenthal & Clarke could not have been estimated satisfactorily in such preparations.

A further contribution to the study of the S-distribution in proteins was made by Baernstein [1936, 1, 2] who had earlier [1932; 1934] exploited Barger & Coyne's [1928] adaptation of the Zeisel procedure for the estimation of methionine. Baernstein found that in the course of digestion with conc. HI cystine was mainly reduced to cysteine and methionine was largely converted into methyl iodide and the thiolactone of homocysteine. Having established that the thiolactone ring would open and reduce tetrathionate to thiosulphate at mild alkalinity, he was able to devise iodine titrations for the thiolactone as well as for the cysteine. The scheme does not permit any discrimination between cystine and cysteine. He found also that SO_4 was reduced to H_2S and SO_2 by digestion with the HI . On this basis he claimed that almost all the S in a variety of proteins occurred in cystine, cysteine, methionine and SO_4 , a claim since disputed by Bailey [1937]. From tests with mixtures of cystine, methionine, other amino-acids and small amounts of glucose, Baernstein believed that the procedure would be applicable without error to impure proteins.

It was found that Baernstein's [1936, 1] methods required modification before they could be applied at all satisfactorily to the impure leaf proteins, particularly in regard to the digestion with HI . His earlier (volatile iodide) method for the estimation of methionine cannot be considered in any way reliable with preparations containing polysaccharide impurity of unknown composition.

Details of procedures and tests

Hydrolysis with HCl. 2 g. samples of preps. 1 and 10 were heated with 15 ml. of 5N HCl at 100° for 24 hr. The mixtures were adjusted to pH 2.5 with NaOH , the insoluble humin was removed and the hydrolysates were adjusted to volume. The *RSSR* [Lugg, 1932] and cystine [Lugg, 1933, 1] contents of the hydrolysates were estimated. The *RSSR* estimations were performed without the aid of a compensating colorimeter to correct for the pronounced adventitious colorations, simply by making enough trials with the HgCl_2 and ferrous salt and then comparing the appropriate "ferrous salt standard" with a cystine standard colour solution. A compensating colorimeter was employed in the single cystine estimation. No *RSH* could be detected with certainty in the hydrolysates, but then no steps had been taken to prevent oxidation to *RSSR*, and in any case the impurities would have left very little *RSH* free.

Digestion with HI. Baernstein's [1936, 1] digestion procedure is to heat for 8 hr. at the boiling point in a current of nitrogen about 0.5 g. of the protein with 10 ml. of fresh, conc. HI (57% HI , sp. gr. 1.7) from which the slight reddish coloration has first been removed by warming with 0.1 g. of KH_2PO_4 . The procedure was tested with mixtures of cystine and methionine, the cystine titrations being about 2% and the methionine about 10% short of stoichiometrical requirements. Similar findings have recently been reported by Kassell & Brand [1938]. With recrystallized edestin the cystine plus cysteine and methionine contents, corrected for the above losses, checked fairly well with the *RSSR* and methionine contents obtained by Bailey [1937] for a different sample of edestin. In the presence of 20% by weight of arabinose, however, the coloration due to iodine was not permanently discharged and there were losses of about 9% of the cystine plus cysteine and about 8% of the methionine; these losses were

increased to about 13 and 19 % respectively (neglecting any effects of modification in the absence of arabinose) by increasing the amount of KH_2PO_4 employed from 0.1 to 0.5 g., which was more than sufficient to prevent the appearance of iodine. Complete elimination of hypophosphite during the 8 hr. digestion period proved to be entirely unsatisfactory, as also was the substitution of red phosphorus for KH_2PO_4 .

The most satisfactory variation was to reduce the coloration of the HI to a deep straw-yellow by heating with the minimum amount of KH_2PO_4 necessary, and to commence the digestion of the protein with the addition of 0.02 g. of KH_2PO_4 , adding like amounts as digestion proceeded and with intervals of not less than 2 min. between additions, so long as the iodine coloration continued to develop. As a rule the intervals are found to be of several hours' duration after the first few additions. It is desirable to avoid an excess of more than 0.02 g. of KH_2PO_4 at any time, and the coloration due to iodine should never be much more than noticeable. With impure leaf proteins the digest can be dark for reasons other than the presence of iodine and care must be exercised in adding KH_2PO_4 . The volume of digest is most satisfactorily reduced later to about 3 ml. by evaporation under reduced pressure in a current of nitrogen with a final 0.02 g. of KH_2PO_4 , and if, on subsequent dilution to 25 ml., the solution is not clear, it should be filtered in a nitrogen atmosphere. A small amount of tarry "humins" develops during the digestion of impure leaf proteins and of arabinose or pectin (especially if protein is present), and an orange coloured flocculum appears when the evaporated digest is diluted. Incidentally, more satisfactory end-points with starch as indicator are obtained in the titration of the cysteine, by adding a slight excess of the iodate solution and instead of back-titrating with the thiosulphate solution, adding a slight excess thereof and back-titrating with the iodate.

Under these modifications mixtures of cystine and methionine showed deficiencies to titration of about 2 % of the cystine and about 5 % of the methionine, as judged by stoichiometrical requirements. Superimposed upon the loss of 2 % of the cystine by titration, some 4 or 5 % of the reduced cystine (cysteine) was modified in such a way (deaminated, decarboxylated?) that it would not react with the highly specific Sullivan reagent. Baernstein [1936, 1] states that the reaction, as used by Sullivan & Hess [1930], may be applied to the cysteine in the diluted HI digests though not very satisfactorily. The reaction was found to be affected by the presence of iodides and the appropriate substances must therefore be introduced in preparing the standard used for colorimetric comparison. Plain HI- KH_2PO_4 digests and HI- KH_2PO_4 digests of gelatin plus tyrosine were used for this purpose, in conjunction with Lugg's [1933, 1] method¹ based upon the Sullivan reaction, and due allowance was made for the minute cystine content of the gelatin. The digests of gelatin plus tyrosine were used when estimating the cysteine contents of protein digests, but were not entirely satisfactory, colour-matching being imperfect.

The effects of the presence of 20 % of arabinose upon the estimations of cystine plus cysteine and methionine in edestin were only slightly reduced by introducing the new digestion procedure (see Table II). The effects of 20 % by weight of "Collier" pectin were about half as great as those of arabinose. There were substantial increases in the values obtained with the impure leaf proteins, however. Blanks with arabinose and pectin were virtually zero.

¹ Strangely enough it has not been recognized by some [e.g. Sullivan & Hess, 1936] that in this method equimolecular amounts of cystine and cysteine yield substantially the same coloration, in contrast to the erratic relationships obtaining in the Sullivan [1926] and Sullivan [1929] methods.

Differential oxidation

SO₄-S after HCl hydrolysis (i.e. without oxidation). 0.5 g. of the preparation was heated at 100° with 5 ml. of 5*N* HCl for 20 hr., and the mixture was adjusted to pH 2.5 with NaOH. After filtering, the filtrate and washings (with 0.01 *N* HCl) were treated with 0.3 ml. of 10*N* HCl and evaporated to 30 ml. on the water bath. 1.0 ml. of 0.5 *M* BaCl₂ solution was added and the solution was kept hot for 1 hr. and then allowed to cool and to stand for 12–15 hr. The BaSO₄ was collected by filtration and washed free from contaminants as judged by a negative chloride test with AgNO₃. It was ignited and weighed in the usual way.

SO₄-S after HNO₃ oxidation. 0.3 g. of the preparation was boiled with 8 ml. of “fuming” HNO₃ (sp. gr. 1.5, 95% HNO₃) for 36 hr. in a 100 ml. round-bottomed flask fitted with a “finger” condenser, and containing a small glass bead to promote even ebullition. The mixture was evaporated with 50 mg. of KNO₃ to dryness on the water bath, and twice re-evaporated to dryness after the addition of 2 ml. of 5*N* HCl each time. It was then taken up in water containing 0.3 ml. of 10*N* HCl and, after filtering, the filtrate and washings were evaporated to 30 ml. and BaSO₄ was precipitated and weighed as before.

Total-S (SO₄-S after peroxide-fusion). 0.2 g. of the preparation was treated with 5 ml. of 1.5 *N* NaOH in a nickel crucible and the mixture was evaporated to dryness at 105°, the drying surface being pricked occasionally to assist evaporation. It was then fused gradually under a mixture of 2 g. each of Na₂O₂ and Na₂CO₃ to a dull red heat, and a further 0.1 g. of Na₂O₂ was added before cooling. The melt was dissolved in warm water, treated with 0.1 g. of Na₂O₂ and acidified slightly by adding 10*N* HCl. After warming to expel free chlorine NaOH was added in slight excess (indicated by the persistence of precipitated nickel hydroxide) and then 10*N* HCl in 0.5 ml. excess over that required to dissolve the nickel hydroxide. The solution was filtered, filtrate and washings were evaporated to 50 ml. and BaSO₄ was precipitated and weighed as before.

All operations were made to conform as far as possible with standard plans, and blanks were determined by putting varying known quantities of Na₂SO₄ through the standardized operations (simple “reagent blanks” can be most misleading).¹ A few checks of the data quoted by Blumenthal & Clarke [1935] for the oxidation of cystine and methionine by HNO₃ proved satisfactory. Addition of 20% by weight of arabinose to edestin was without effect upon the values obtained with the protein itself. The presence of finely divided silica in HNO₃ digests of the impure leaf proteins may have been responsible for their occasional tendency not to boil smoothly.

RESULTS AND DISCUSSION

The protein portions proper of preparations which contain non-nitrogenous impurity are most readily compared in amino-acid composition by referring the analyses to a nitrogen basis. It is believed that none of the preparations contained more than a trace of nitrogenous impurity. The work was done with air-dried materials containing several % of moisture as a rule, but drying at 105° for 18 hr., contrary to Baernstein's [1936, 1] findings, did not affect the estimations detectably.

¹ Iron and aluminium, if present in appreciable quantity, must be removed from solution by the usual methods before precipitating the BaSO₄.

The results of analyses of the leaf protein preparations are shown in Table II, and are prefaced by those of edestin (A) and edestin plus 20% by weight of arabinose (B). Some of the values are from single, others from duplicate and triplicate estimations. The random errors, as % of the values reported,

Table II

No.	% N	HCl hydrolysis		HI digestion			Differential oxidation			
		% cyst.-N (RSSR)	% cyst.-N (sp.)	% cyst.-N (sp.)	% cyst.-N (titr.)	% meth.-N (titr.)	% cyst.-N	% meth.-N	% cyst.-N + % meth.-N	SO ₄ -S × 100/N
A	18.5	0.88*	0.82*	0.87	0.90	1.18	0.94	1.24	2.18	0.07
B	(15.4)	0.59†	0.59†	—	0.84	1.09	0.94	1.24	2.18	0.07
1	13.1	0.14	—	—	1.38	1.21	1.51	1.44	2.95	0.33
2	13.0	—	—	—	1.28	1.20	1.47	1.35	2.82	0.27
3	13.1	—	—	—	1.28	1.19	1.53	1.47	3.00	0.19
4	14.1	—	—	1.4	1.50	1.30	1.68	1.49	3.17	0.40
5	15.4	—	—	1.5	1.50	1.45	1.63	1.48	3.11	0.52
6	15.35	—	—	1.4	1.40	1.36	1.58	1.44	3.02	0.89
7	13.85	—	—	1.1	1.11	1.22	1.19	1.23	2.42	0.29
8	13.85	—	—	—	—	—	1.31	1.23	2.54	0.34
9	14.2	—	—	—	1.21	1.31	1.29	1.35	2.64	0.41
10	12.1	0.74	0.52	1.15	1.30	1.55	1.44	1.53	2.97	1.92

Note. Quantities marked * are recalculated, with corrections for losses of the free amino-acids, from Bailey's [1937] data for a different sample of edestin containing 18.4% N but prepared from the same batch of hemp seed by the same method, and those marked † for edestin to which 10% by weight of arabinose had been added. Bailey found 20% loss of methionine after hydrolysing the edestin-arabinose mixture with HCl and removing the humin, and no loss in absence of arabinose, the % methionine-N by Baernstein's [1934] "volatile iodide" procedure being 1.23 (recalculated with correction). Baernstein [1936, 2] reports values by titration of HI digests for two samples of edestin, the values for one of the samples agreeing fairly well with those shown above in the table.

are believed not to exceed those indicated below in the description of the contents of the table. In the order of the columns are shown: the number or letter distinguishing the preparation (see Table I): the % N in it (error 0.5%, affecting all the other errors); and then a series of estimated cystine plus cysteine and methionine contents expressed as % of the total N appearing as these amino-acids and a few quantities incidental thereto. The series is: cystine plus cysteine from RSSR content (error 2% for A, 5% for the others), and by specific colorimetric estimation (error 3% for A, 5% for the others) of HCl hydrolysates; cystine plus cysteine by specific colorimetric estimation (error 4 or 5% for A, 8% for the others), cystine plus cysteine from titration (error 1 or 2% for A and B, 3% for the others), and methionine from titration (error 2% for A and B, 4% for the others) of HI digests; cystine plus cysteine (error 2% for A and B, 4% for the others) and methionine (error 3% for A and B, 5% for the others) from differential oxidation on the assumption that these are the only S-containing amino-acids and that their S appropriately appears as SO₄ in the successive stages of oxidation; the sum of the last two values (error 2%); and finally the SO₄-S as % of the weight of N in the material taken (error from 2% for the larger to 20% for the smaller values). All values have been corrected only for the losses to which the free amino-acids are subject in the procedures involved, and even these corrections, though small, are not rigorously applicable.

In the first place it will be noticed that the various procedures all lead to substantially the same values in the case of edestin (A), and in the second place that the values obtained with the impure leaf proteins vary with procedure in a manner reminiscent of the effects of adding arabinose to edestin (B). The

extremely low *RSSR* content of the HCl hydrolysate of 1 recalls similar low cystine contents of cocksfoot leaf protein HCl hydrolysates reported by Pollard & Chibnall [1934]. From various considerations (extensive amino-acid analyses, the amounts of humin produced on acid hydrolysis etc.) it seems that the cocksfoot preparations, and probably those from other species of leaf too, should contain rather more than 16.5% N if they were "pure", as against an earlier computation by Miller [1936] of 16.0%, and that the usual organic impurity may be a pentosan, a pectin or a mucilage with the evidence mostly in favour of the last. There can be little doubt therefore that the major variations of the values with procedure, as shown in Table II, are due to the presence of organic impurities.

A greater amount of impurity may be expected to have a greater effect upon an estimation, and the degrees of vitiation may well depend upon the precise nature of the protein as well as that of the impurity. In the case of edestin itself (A), however, there are definite, small variations with procedure, and these may be attributed either to the presence of very small amounts of other S-containing amino-acids or to the deficiencies of methods of estimation which still involve the unwarrantable assumption that an amino-acid residue in protein behaves precisely as does the corresponding free amino-acid. Moreover, although the disparities between the values obtained by HI digestion and differential oxidation of the impure leaf proteins are generally greater than they are with edestin plus arabinose, it is justifiable to assume that the impurities would cause some loss in the former procedure. The differential oxidation procedure therefore, though less specific, may yield the more appropriate values both with edestin and with the impure leaf proteins. Complete acceptance of these values would imply an acceptance of the provisional assumptions as to the nature of the S fractions, recognition that the sum of the fractions thus estimated necessarily equals the estimated total S and a denial of the existence of systematic errors. At all events it seems extremely unlikely that the real cystine plus cysteine contents could have been appreciably lower than those obtained by HI digestion, because titration and colorimetric estimations check well.

Any doubt concerning the specificity of the differential oxidation procedure notwithstanding, the values obtained by it are of use in deciding whether one protein differs from another in amino-acid composition. The individual cystine plus cysteine and methionine values lack the precision which their sums possess, but these too may be used for the purpose, and it can be stated that the preparations made from one species of leaf were not all identical in composition, even when prepared from the same batch of leaf material.

The three preparations from lucerne leaves were of slightly lower cystine plus cysteine and methionine contents than the other leaf protein preparations were, but by the usual standards of animal nutritional requirements none could be adjudged deficient in its contents of these amino-acids.

SUMMARY

The preparation of some samples of protein from the fresh leaves of cocksfoot grass, Toowoomba canary-grass, lucerne and Oldman salt-bush, is described.

Methods of determining the S-distributions of the samples are discussed and described and the results of analyses are recorded.

It is pointed out that as the cystine (and/or cysteine) contents appear to lie within the range 1.1–1.7%, and the methionine within the range 1.2–1.6% of the protein-N, none of the preparations can be regarded as deficient in these amino-acids by the usual standards of the nutritional requirements of animals.

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